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Overexpression of IAP-2 attenuates apoptosis and protects against myocardial ischemia/reperfusion injury in transgenic mice

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Abstract

Inhibitors of apoptosis proteins (IAPs) are key intrinsic regulators of caspases-3 and -7. During ischemia, IAP-2 is upregulated dramatically, while the other IAPs show little or no change. To test whether IAP-2 prevents cardiac apoptosis and injury following ischemia/reperfusion, we generated a line of transgenic mice that carried a mouse IAP-2 transgene. High levels of mouse IAP-2 transcripts and 70 kDa IAP-2 were expressed in the hearts of transgenic mice, whereas IAP-1 and XIAP levels remained the same. Immunohistochemical studies revealed more intense staining of IAP-2 in the myocytes of transgenic mouse hearts. To assess the role of IAP-2 in I/R injury, the transgenic mice were subjected to ligation of the left descending anterior coronary artery ligation followed by reperfusion. The infarct sizes, expressed as the percentage of the area at risk, were significantly smaller in the transgenic mice than in the non-transgenic mice (30±2% vs. 44±2%, respectively, $P<0.05$). This protection was accompanied by a decrease of the serum level of troponin I in the transgenic mice. IAP-2 transgenic hearts had significantly fewer TUNEL-positive cardiac cells, which indicated an attenuation of apoptosis. Our results demonstrate that overexpression of IAP-2 renders the heart more resistant to apoptosis and I/R injury.

Keywords: Caspases; Inhibitor of apoptosis proteins; Ischemia/reperfusion injury; Heart

1. Introduction

Apoptosis has been implicated in a number of cardiac diseases such as heart failure [1–3], anthracycline-induced cardiotoxicity [4–6], and overstretching of myocytes [7]. Myocardial ischemia/reperfusion (I/R) also leads to cell death, which is believed to occur through apoptosis and necrosis [8–11]. Kajstura et al. [12] showed that apoptosis was the predominant mode of cardiac cell death induced by coronary artery occlusion.

There are three main pathways leading to apoptosis [13–15]. The extrinsic apoptotic pathway is mediated by the death receptor Fas/FasL and involves the activation of caspase-8. The intrinsic pathway involves mitochondrial dysfunction, cytochrome *c* release, and activation of caspase-9. The third apoptosis pathway is activated by ER stress and involves caspase-12.

Caspases are the major players for the execution of apoptosis [16,17]. They can be categorized into initiator caspases (-2, -8, -9, -10, and -12) and executioner caspases (-3, -6, and -7). Initiator caspases undergo autoproteolytic activation, while executioner caspases are responsible for dismantling cellular structure. Activation of various caspases can be blocked by inhibitor of apoptosis proteins (IAPs). IAP family members are characterized by the presence of one or more BIR domains in their sequence and by their ability to bind and inhibit caspases. Eight IAP members have been discovered so far, namely IAP-1, IAP2, XIAP, ILP2, MLIAP, NIAP, survivin, and Bruce [18–24]. Recent studies demonstrate that XIAP, IAP-1, and IAP-2 can prevent the proteolytic processing of procaspases-3, -6, and -7

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by blocking the cytochrome *c*-induced activation of procaspase-9 [25].

IAP-2 has been detected in the heart, but its physiological role is not clear [26]. To further understand the role of IAP-2 in myocardial I/R injury and apoptosis in a more physiological setting, an animal model that overexpresses IAP-2 was needed. Toward this end, our experiments were designed to achieve the following goals: (1) to generate transgenic mice bearing extra copies of cloned mouse IAP-2 cDNAs under the transcriptional control of a mouse α-myosin heavy chain promoter to allow high-level expression of transgenes in the heart; (2) to determine the levels of expressed IAP-2 in the hearts of these animals; and (3) to elucidate the effect of IAP-2 overexpression on ischemia/reperfusion injury and apoptosis.

2. Materials and methods

2.1. Generation of IAP-2 transgenic mice

An IAP-2 expression vector was constructed by initially inserting the *Sac*I to *Sal*I fragment of clone 22 (kindly provided by Dr. J. Robbins, University of Cincinnati, Cincinnati, OH), which contains the sequence from the last intron of the mouse β-myosin heavy chain gene to exon 3 of the α-myosin heavy chain gene, into *Sac*I to *Sal*I sites in plasmid pMSG (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). *Bam*HI digestion of the resultant plasmid allowed isolation of the DNA fragment containing SV40 early splicing and polyadenylation sites downstream from the mouse α-myosin heavy chain sequence. This DNA fragment was then inserted into the *Bam*HI site of plasmid pKS-S, a modified pKS vector (Stratagene, La Jolla, CA) in which the *Sal*I site was destructed by insertion of an *Sfi*I linker, to generate plasmid pMHC. The full-length mouse IAP-2 cDNA, which had previously been flanked by *Sal*I sites using linker ligation, was subsequently inserted into the *Sal*I site in plasmid pMHC. The entire expression sequence was isolated by *Cla*I plus *Not*I digestion of the resultant plasmid, and it was utilized in the generation of transgenic mice using fertilized mouse eggs isolated from mating of B6C3 F1 hybrid mice according to standard procedures.

2.2. Analysis of in vivo cardiac function

Mice were anesthetized with tribromoethanol (275 mg/kg, i.p.). Each mouse was intubated with a 22-gauge soft catheter and ventilated with a rodent ventilator (Columbus Instruments International Corp., Columbus, OH) at a tidal volume of 0.3–0.5 ml and a respiratory rate of 110–120 breaths/min. After left thoracotomy, the pericardium was dissected to expose the heart. A 26-gauge needle connected to a pressure transducer was introduced into the left ventricle after an apical stab to measure the left ventricular pressure. Cardiac function parameters, including LVEDP (left ventricular end-diastolic pressure), LVESP (left ventricular end-systolic pressure), $\pm dP/dt$ (first derivatives of left ventricular pressure over time), were analyzed with a SonoSoft data acquisition and analysis system (SonoMetrics Corp., London, Ontario).

2.3. Microarray analysis

Total RNA was extracted from the hearts of nontransgenic and transgenic mice using TRI REAGENT LS (Molecular Research Center, Cincinnati, OH). Superarray analysis was carried out according to manufacturer's instructions (Superarray, Inc., Frederick, MD). cDNA probes were labeled with Ampolabeling-LPR kit in the presence of 5 μg of RNA and biotin-16-dUTP. Labeled probes were hybridized to mouse apoptosis GEArray Q series membranes. After extensive washing, the chemiluminescence signals on the membranes were exposed to X-ray films. Quantification was performed with optimally exposed films and standardized against the housekeeping gene GAPDH using the GEarray Analysis Suite.

2.4. Immunoblot analysis of IAP-1, IAP-2, and XIAP

Hearts from nontransgenic and transgenic animals were homogenized in a buffer containing 20 mM Tris–HCl (pH 7.8), 137 mM NaCl, 15% glycerol, 1% Triton X-100, 2 μg/ml each of leupeptin, aprotinin, and pepstatin, 2 mM benzamidine, 20 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 25 mM beta-glycerophosphate, and 0.1 mM phenylmethylsulfonylfluoride. Homogenates were heated at 95 °C for 10 min, passed through a 23 gauge needle five times, and centrifuged at 12,000×*g* for 10 min. Protein concentration of the supernatant was determined by Biorad dye binding assay. Aliquots containing 100 μg were electrophoresed on 12% SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblot analysis was carried out by incubating the membrane with IAP-1, IAP-2 (Santa Cruz Biotechnology, Santa Cruz, CA), or XIAP antibodies (BD-Pharmingen, San Diego, CA). Horseradish peroxidase conjugated goat anti-rabbit or anti-mouse antibodies were then added. Blot was developed using ECL detection system (Amersham Biosciences, Piscataway, NJ) and exposed to X-ray films.

2.5. Immunohistochemistry

Paraffin-embedded myocardial sections (5 μm) were mounted on superfrost slides and dried at 37 °C overnight. Heart specimens were subjected to antigen retrieval by heating in 10 mM citrate (pH 6.0) in a microwave oven at 100% power for 3.5 min and 50% power for 8 min. Immunostaining was carried out with a rabbit IAP-2 antibody (R&D Systems, Minneapolis, MN) at 4 °C overnight. Antigen–antibody complexes were detected by the Super-sensitive alkaline phosphatase kit (BioGenex, San Ramon, CA), using Fast Red as a chromogen. Hematoxylin was used as a counterstain.

2.6. Regional ischemia in vivo

Mice weighing 25–30 g were anesthetized with tribromoethanol (275 mg/kg, i.p.). A 22-gauge soft catheter was inserted 5–8 mm from the larynx, and the mice were ventilated with room air (a tidal volume of 0.3–0.5 ml) using a rodent respirator (Columbus Instruments International, Columbus, OH) set at 110–120 beats/min. Left anterior descending coronary artery (LAD) ligation was performed as described previously [27]. After 50 min of LAD ligation, the heart was reperfused for 4 h.

Mice were anesthetized with tribromoethanol (275 mg/kg, i.p.), and hearts were perfused as Langendorff preparations for 5 min. The left coronary artery was re-occluded, and 1% Evans blue was infused into the aorta and coronary arteries to determine the area at risk. Hearts were transversely cut into 5 sections, with one section made at the site of the ligature. Macroscopic staining with 1% triphenyltetrazolium chloride (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline was used to quantitate the infarct sizes as described previously [27]. The area at risk was expressed as the percentage of the left ventricle, and the area of infarct was expressed as the percentage of the area at risk as described [27].

2.7. Cardiac troponin I release

Although LDH activity has been used as a biomarker of cardiac injury, it is not tissue-specific and the enzyme activity is rather unstable upon freezing and thawing. As a result, an ELISA kit (Life Diagnostics, Inc., West Chester, PA) was used to assay the plasma levels of cardiac troponin I, which has been shown to be a more sensitive and specific biomarker of cardiac injury. Results were expressed as ng/ml.

2.8. TUNEL assays

After 50 min of LAD ligation and 4 h of reperfusion, hearts were removed, and a 2 mm section near the middle part of the area at risk was sliced, fixed in 4% formalin solution and embedded in paraffin. Myocardial sections (5 μm) were mounted on superfrost slides and dried at 37 °C overnight. Immunohistochemical procedures for detecting apoptotic cardiomyocytes were performed

by using CardioTACS™ (Trevigen, Inc. Gaithersburg, MD) as described previously [27]. In this procedure, nuclei undergoing apoptosis were stained blue. Nuclear Fast Red was used as a counterstain. TUNEL-positive myocytes were determined by randomly counting 10 fields of approximately 1000 nuclei. Myocyte nuclei are characterized by their size, shape, and location. Counting was performed in a blinded fashion. The index of apoptosis was then determined (i.e., number of apoptotic myocytes/total number of myocytes counted × 100).

2.9. Statistical analysis

Statistical analysis was performed by the Instat software. Significance of differences between means was established by the Student's *t* test. Results were expressed as means ± SEM. $P < 0.05$ was considered significant.

3. Results

We generated a line of transgenic mice that carried a mouse IAP-2 transgene under the control of a mouse α-myosin heavy chain promoter. Table 1 summarizes the basal hemodynamic indices (HR, LVEDP, LVESP, and ±d*P*/d*t*) of the nontransgenic and transgenic mice. At the age of 3–4 months, there were no differences in heart weight, body weight, heart weight/body weight or the basal cardiac function between the nontransgenic and transgenic mice.

We performed a detailed gene expression study with the offspring of the founder mouse. Total heart RNA was isolated from nontransgenic or transgenic animals and subjected to gene array analysis using Superarray mouse apoptosis Q-series membranes. This allowed us to study the expression of 96 mouse apoptosis-related genes. There were a few genes that were upregulated or downregulated in the IAP-2 transgenic heart. The most apparent change was the upregulation of IAP-2 mRNA by 2.5-fold (Fig. 1).

We next determined IAP-2 protein expression by Western blot analysis. Our results showed that the 70 kDa IAP-2 was highly expressed in the transgenic heart (Fig. 2). Densitometric analysis demonstrated a 2.3-fold increase of IAP-2 after standardization with actin. Overexpression of IAP-2 did not induce any changes in the expression of IAP-1 or XIAP (Fig. 2).

Immunoreactivity of IAP-2 demonstrated that it was strongly expressed in the myocytes of transgenic hearts, as demonstrated by diffused cytoplasmic staining. Endothelial cells or smooth muscle cells did not show immunoreactivity to IAP-2 antibody. There was significantly less IAP-2 staining in the nontransgenic heart (Fig. 3).

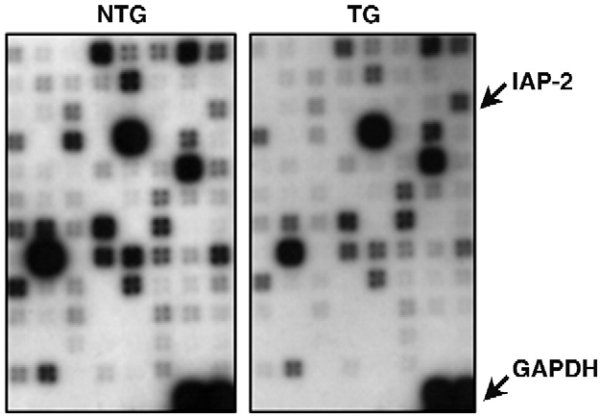

Fig. 1. IAP-2 mRNA is overexpressed in the transgenic mouse heart. RNA was isolated from the hearts of nontransgenic and transgenic animals. Gene array analysis was performed with mouse apoptosis Superarray Q series membranes as described in Materials and methods. The position of IAP-2 and GAPDH is marked by arrows.

To study the effect of IAP-2 overexpression on regional I/R injury *in vivo*, mice were subjected to 50 min of LAD ligation followed by 4 h of reperfusion. Fig. 4A shows a representative TTC staining pattern of nontransgenic and transgenic hearts. Infarcted areas appear pale, whereas viable tissues stain red. The area at risk, expressed as the percentage of the left ventricle (LV) between the nontransgenic and transgenic hearts, was comparable (34 ± 1.6% vs. 33 ± 1.7%, respectively). Infarct sizes of the nontransgenic hearts and transgenic hearts were 15 ± 0.5% and 9 ± 0.5% of the LV, respectively. Infarct sizes, expressed as the percentage of the area at risk for the nontransgenic and transgenic hearts, were 44 ± 2% and 30 ± 2%, respectively (Fig. 4B). Our results indicate that overexpression of IAP-2 is able to limit the infarct size in an *in vivo* regional ischemia model.

Cardiac troponin I release has been used as a marker for cardiac injury. We did a pilot study to follow troponin I release

Table 1
Basal cardiac function of nontransgenic and transgenic mice

Parameter	Nontransgenics	Transgenics
Heart Rate (beats/min)	364 ± 10	363 ± 21
+d*P*/d*t* (mm Hg/s)	2899 ± 75	3381 ± 477
−d*P*/d*t* (mm Hg/s)	2329 ± 105	2565 ± 191
LVDP (mm Hg)	81 ± 1.7	95 ± 4
LVEDP (mm Hg)	3.8 ± 0.7	3.0 ± 0.7
Heart wt, mg	86.4 ± 2.3	87.2 ± 4.9
Body wt, g	32 ± 2.5	29 ± 1.0
Heart wt/body wt (mg/g)	2.8 ± 0.2	3.0 ± 0.2

In vivo cardiac function was measured by the SonoMetrics system as described in Materials and methods. All values are means ± SEM of 5–6 hearts. Cardiac functions are defined as: HR, heart rate, d*P*/d*t*, maximum rate of pressure development; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure.

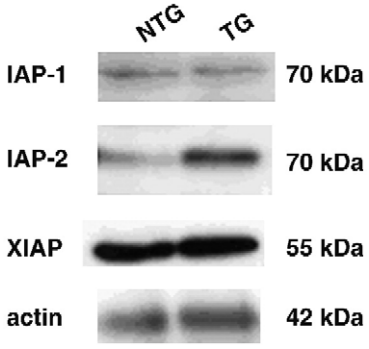

Fig. 2. Western blot analysis of IAP-1, IAP-2, and X-IAP. Heart homogenates of nontransgenic and transgenic mice were probed with specific antibodies against IAP-1, IAP-2, and XIAP. Actin antibody was included to check protein loading.

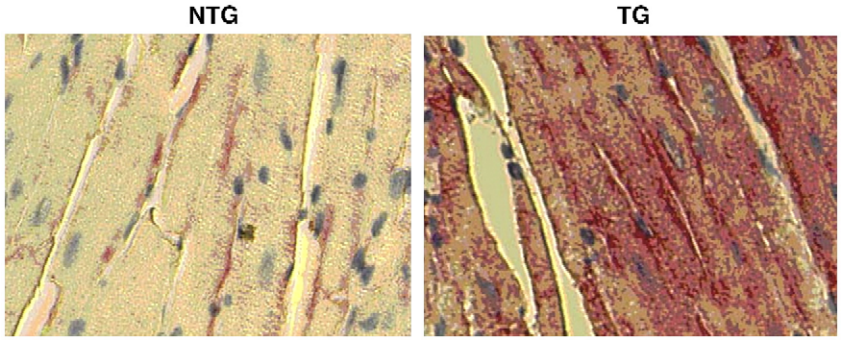

Fig. 3. Immunohistochemical staining of IAP-2 in hearts of nontransgenic and transgenic mice. Paraffin-embedded sections of nontransgenic and transgenic heart were immunostained with an IAP-2 antibody. IAP-2 immunoreactivity (in red) was demonstrated by diffused cytoplasmic staining in the myocytes in panel B. Magnification, ×200.

in the nontransgenic mice after reperfusion. As shown in Fig. 5A, troponin I release significantly increased after 1 h of reperfusion and reached a maximal level after 3–4 h. We next compared troponin I level in the sera of nontransgenic and transgenic animals after 4 h of reperfusion. The total release of cardiac troponin I of the nontransgenic and transgenic group was 108±9 ng/ml and 64±9 ng/ml ($P<0.05$), respectively, indicating a 1.7-fold reduction (Fig. 5B).

To examine whether the functional protection of the IAP-2 transgenic hearts is related to the anti-apoptotic property of IAP-2, TUNEL analyses were performed on hearts subjected to 50 min of LAD ligation and 4 h of reperfusion. A representative staining pattern is shown in Fig. 6A. Heart tissue from sham-operated nontransgenic and transgenic mice exhibited low levels of staining for TUNEL (1.8±0.45%, $n=6$) and (1.0±0.15%, $n=6$), respectively (Fig. 6B). TUNEL-positive myocytes from

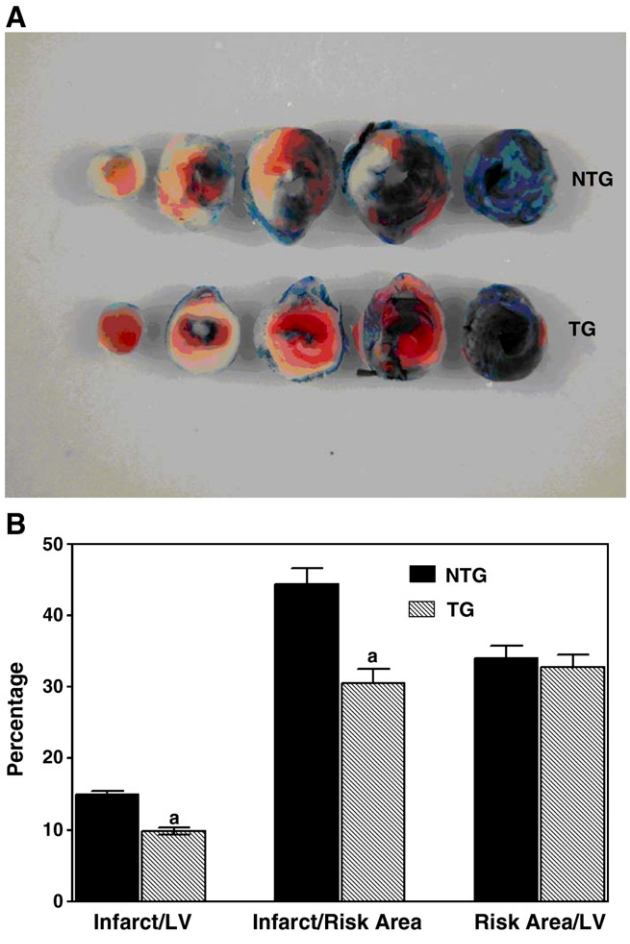

Fig. 4. Protective effect of IAP-2 overexpression on myocardial infarction in mice. Animals were subjected to 50 min of LAD coronary artery ligation followed by 4 h of reperfusion. Panel A represents the TTC-staining pattern of nontransgenic and transgenic hearts, respectively. Panel B shows an analysis of the effect of IAP-2 overexpression on infarct sizes. Values are means±SE of 6 hearts. a=$P<0.05$ vs. nontransgenics.

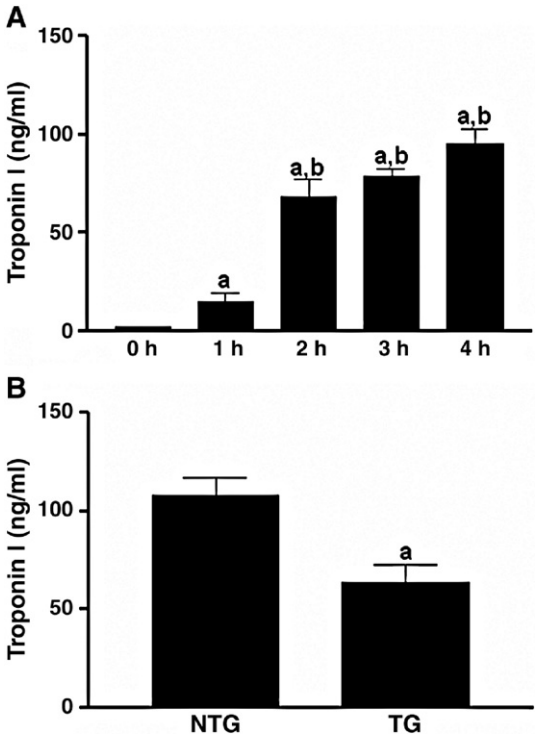

Fig. 5. Release of cardiac troponin I. Mice were subjected to 50 min of LAD coronary artery ligation followed by 4 h of reperfusion. Serum troponin I was measured by an ELISA kit. Values represent means±SE. a=$P<0.05$ vs. nontransgenics.

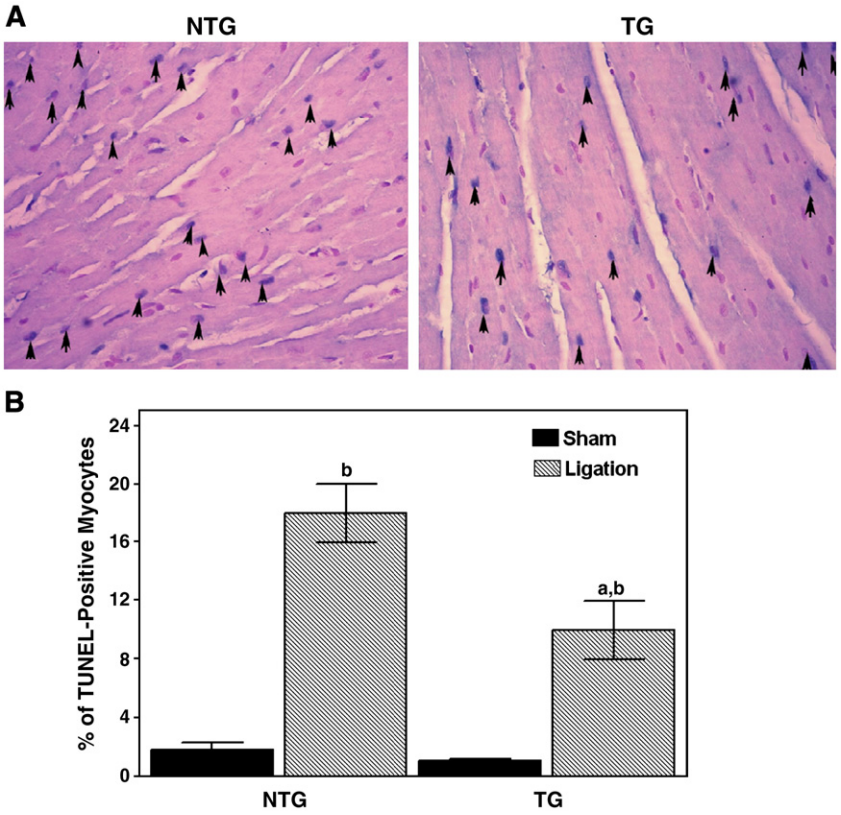

Fig. 6. Detection of apoptosis by the TUNEL procedure. Paraffin-embedded sections of nontransgenic and transgenic hearts subjected to 50 min of LAD coronary artery ligation followed by 4 h of reperfusion were stained by TUNEL. Representative staining pattern of nontransgenic and transgenic hearts is shown in A. Arrows indicate TUNEL-positive nuclei which were stained blue. Magnification, ×200. TUNEL-positive nuclei of myocytes were determined by random counting of 10 fields per section. Each bar represents means±SE of six hearts. a=$P<0.05$, LAD ligation group in TG vs. NTG, b=$P<0.05$ vs. sham group (B).

the left ventricles of nontransgenic animals and transgenic animals were (18.0±0.45%, $n=6$) and (10.0±2.0%, $n=6$, $P<0.05$), respectively.

4. Discussion

There are two major types of anti-apoptotic proteins: Bcl-2 family proteins and IAPs. Previously, we reported that overexpression of human Bcl-2 gene renders the mouse heart more resistant to apoptosis and I/R injury [27]. In the present study, we demonstrate that overexpression of IAP-2 confers cardioprotection against I/R injury *in vivo*. In our study, transgenic hearts had fewer TUNEL-positive cardiomyocytes than nontransgenic hearts, suggesting that the cardioprotective effect of IAP-2 is related to inhibition of cardiac apoptosis.

The finding that IAP-2 can protect the heart against I/R injury is in line with previous studies on the IAP family. First, IAP-2 level is known to be regulated by hypoxia [28]. Second, overexpression of IAP-2 inhibits apoptosis induced by Fas and serum withdrawal in several cell systems [25]. Finally, overexpression of NIAP and XIAP in the brain reduces ischemic damage in the rat hippocampus [29] and protects against the toxicity of dopaminergic neurotoxin MPTP [30]. Our results are also in agreement with previous studies that show that caspase inhibition by general caspase inhibitors reduces infarct size and inhibits apoptosis [31–33]. For example, Holly et al. [31] reported that systemic administration of a broad spectrum caspase inhibitor partially attenuated caspase activation, decreased TUNEL-positive myocytes, and reduced cardiac infarct size.

The mechanism of the cardioprotective effect of IAP-2 is not clear, but there are a number of different possibilities. IAP-2 inhibits active caspases-3 and -7 and blocks the activation of procaspase-9 [25]. In addition, IAP-2 can suppress caspase-8 activation [25,34,35]. These studies suggest that IAP-2 suppresses both intrinsic and extrinsic apoptotic pathways by inhibiting specific caspases.

However, accumulating evidence indicates that IAPs can inhibit apoptosis through mechanisms other than caspase inhibition. First, IAPs can interact with TRAF-1 and TRAF-2 and play a role in type 2 TNF receptor signaling [36]. Second, IAP-2 possesses a RING domain that functions as ubiquitin E3 ligase [37]. The degradation targets include caspases-3, -7, and death-inducing protein SMAC (second mitochondria-derived activator of caspases) [38–40]. Finally, IAP-2 may exert its protective effect through the activation of NF-κB [41]. In short, IAP-2's cardioprotective effect may be mediated by caspase inhibition, protein degradation, and regulation of signaling pathways. The precise mechanism of the cardioprotective effect requires further investigation.

It is believed that there is significant interaction between the IAP family members. For example, in XIAP deficient mice, levels of IAP-1 and IAP-2 are increased [42]. In addition, IAP-1

knockout mice exhibit elevated levels of IAP-2 [43]. Given the possible interactions between IAP family members, it is possible that in our transgenic mice, overexpression of IAP-2 affects other IAPs. However, our Western blot analysis demonstrated that there were no alterations in the IAP-1 or XIAP levels in the hearts of IAP-2 overexpressed mice (Fig. 2). This was also confirmed by immunohistochemical staining (results not shown).

One limitation of the present studies is that it does not address whether reduction of infarct size in the IAP-2 hearts correlates with improved cardiac function. We have shown previously that overexpression of the anti-apoptotic protein Bcl-2 reduced infarct sizes and improved cardiac function (27). However, future studies will be needed to address whether this is also true with IAP-2.

In summary, overexpression of IAP-2 protects the heart against I/R injury and inhibits cardiac apoptosis. Though the exact mechanism of these effects remains unclear, these findings suggest that IAP overexpression strategies may be used to prolong cardiomyocyte survival.

Acknowledgments

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